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Embryo sac development is affected in *Petunia inflata* plants transformed with an antisense gene encoding the extracellular domain of receptor kinase PRK1

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Abstract In a previous study of the function of a pollen-expressed receptor kinase of *Petunia inflata*, PRK1, it was found that transgenic plants carrying an antisense-*PRK1* gene were unable to transmit the transgene through either the male or, unexpectedly, the female. In this report, the nature of this female phenotype was studied using one of the transgenic plants, ASRK-13. Electron and light microscopic examination of the embryo sac and seed development of ASRK-13 and a wild-type plant revealed that embryo sac development of approximately half of the ovules of ASRK-13 was abnormal. The development of the affected embryo sacs was arrested at the late stages of megagametogenesis. The majority of the affected embryo sacs completed three rounds of mitosis normally, but failed to progress through the maturation stages when cell expansion, nuclear migration, and differentiation take place. The remaining small number of abnormal embryo sacs were arrested at either the four- or eight-nucleate stages. The ovules containing the defective embryo sacs apparently failed to be fertilized, resulting in degeneration of half of the seeds produced by ASRK-13. RNA gel blot analysis suggests that the *PRK1* gene is expressed in the ovary, albeit at a much lower level than in the anther. The possibility that the antisense *PRK1* gene is responsible for the abnormal embryo sac development is discussed.

Key words Embryo sac development · Ovule · *Petunia inflata* · Receptor kinase

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Introduction

Receptor kineses play important roles in cell-cell signaling, which is required for normal growth and development in animals (Pawson and Bernstein 1990; Ullrich and Schlessinger 1990; Meek and Street 1992). Genes encoding receptor kineses from various plant species have been reported (Walker and Zhang 1990; Stein et al. 1991; Chang et al. 1992; Walker 1993; Mu et al. 1994; Song et al. 1995; Becraft et al. 1996; Torii et al. 1996), and the functions of some of these plant receptor kineses have been determined. Among them, SRK of *Brassica oleracea* is involved in self/non-self recognition between pollen and pistil during self-incompatibility interactions (Stein et al. 1991; Goring et al. 1993; Nasrallah et al. 1994; Delorme et al. 1995; Shiba et al. 1995); Xa21 of rice mediates resistance to a particular race of bacterial pathogen *Xanthomonas oryzae* (Song et al. 1995); PRK1 of *Petunia inflata* (Mu et al. 1994) is essential for post-meiotic development of pollen (Lee et al. 1996); ERECTA of *Arabidopsis thaliana* is required for development of organs derived from shoot meristem (Torii et al. 1996); and CRINKLY4 of maize is involved in leaf epidermis differentiation (Becraft et al. 1996).

When studying the function of PRK1, we constructed an antisense *PRK1* gene that contained a 0.6-kb *SacI-Bam*HI cDNA fragment, encoding approximately 58% of the extracellular domain of PRK1 (Mu et al. 1994), fused to the *LAT52* promoter of tomato (Twell et al. 1990) in

antisense orientation, and used this construct to transform *P. inflata* plants. In three transgenic plants, ASRK-13, ASRK-20, and ASRK-59, development of approximately half of the microspores was arrested prior to the first microspore mitosis (Lee et al. 1996). The arrested microspores then lost most of their cytoplasmic contents. Consequently, when pollen from these transgenic plants was used to pollinate wild-type plants, the antisense transgene did not transmit to the progeny. Unexpectedly, when reciprocal crosses were carried out between these transgenic plants and wild-type plants, transmission of the antisense transgene to the progeny through the female was also greatly reduced. For example, only one of the 132 progeny of the transgenic plant ASRK-13 and seven of the 95 progeny of the transgenic plant ASRK-20 were found to carry the transgene (Lee et al. 1996). These results suggest that PRK1 may play a role in ovule development, pollen tube/ovule interactions, fertilization, or postzygotic development (embryogenesis and endosperm development).

As a first step towards addressing the possible function of PRK1 in any of these processes, we set out to characterize the embryo sac and seed development of one of the transgenic plants, ASRK-13, and a wild-type plant to identify the developmental process that was affected in ASRK-13. Here, we report these results, along with our preliminary characterization of the expression of the *PRK1* gene in the ovary.

Materials and methods

Differential interference contrast microscopy

Ovaries from open flowers were fixed for 24 h in a fixing solution composed of absolute ethanol, acetic acid, 37% formaldehyde, and H_2O (50:5:10:35, v:v:v:v). After rinsing with 50% ethanol three times, the ovules were dissected from each ovary and incubated in Herr's clearing fluid (Herr 1971) composed of 80% lactic acid, chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1, w:w:w:w:w) for 24 h at room temperature on a microslide. After clearing, the ovules were examined under a differential interference contrast microscope.

Scanning electron microscopy and light microscopy

For scanning electron microscopy (SEM), freshly collected ovaries were fixed for 24 h in the fixing solution described above. After washing with 70% ethanol, the pericarp was removed from the ovary to expose the ovules. The tissues were incubated in 90% ethanol for 30 min, then in absolute ethanol for 30 min twice. After critical point drying in a critical point dryer (Bio-Rad/EBS, Agawam, Mass., USA), the samples were gently placed on stubs coated with double sticky tape and coated with 10 nm gold-palladium with a sputter coaler (BAL-TEC, Boston, Mass., USA) Observations were made with a JEOL JSM5400 scanning electron microscope. For light microscopy of longitudinal sections of ovaries, ovaries were fixed in 1.5% gluteraldehyde and 2.5% paraformaldehyde for 14 h at room temperature under vacuum. The samples were then washed three times with 0.1 M phosphate buffer (PB), pH 7.4, and fixed in 1% osmium tetroxide in PB for 5 h at room temperature. After washing in several changes of PB, the tissues were dehydrated with a graded ethanol series (from 50% to 100%). The ovaries were then stained in 2% uranyl acetate in 100% ethanol at 4°C overnight, washed in 100% ethanol and then 100% propylene oxide, and infiltrated with Spurrs medium (EM Sciences, Gibbstown, New Jersey, USA) and propylene oxide (50%/50%, 75%/25%, 100%, 100% for 8 h each). The resin was polymerized for 12 h at 70°C. Thin sections were prepared with an LKB III-8800 ultramicrotome and observed on a Dialux 20 EB microscope.

Staining of pollen tubes in ovules

Ovaries at 2 days after pollination (DAP) were fixed for 24 h in the fixing solution described for differential interference contrast microscopy. After fixation, the pericarp of the ovary was removed to expose the ovules. After hydration through a graded ethanol series (from 50% to 0% ethanol), the tissues were incubated in 10 N NaOH for 2 h at room temperature. After washing with distilled water for 30 min twice, the ovaries were stained in 0.015% toluidine blue (neutral pH) for 15 min, washed in distilled water for 1 h, and stained in 0.1% aniline blue (in 2% K_3PO_4, pH 10–12) for 1 h. The whole ovary in a drop of glycerol was then gently squashed onto a microslide and observed under a fluorescence microscope.

RNA gel blot analysis

Total RNA was isolated from 1 g each of anther and ovary tissues as described previously (Kheyr-Pour et al. 1990). Poly $(A)^+$ RNA was isolated from total ovary RNA using a poly (A) tract mRNA isolation kit (Promega, Madison, Wis., USA). RNA samples were electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to a Hybond N membrane (Amersham, Arlington Heights, Ill., USA). The membrane was pre-hybridized in ×5 SSC, ×5 Denhardt's solution and 0.5% SDS at 6°C for 2 h and hybridized overnight in the same solution containing a ^{32}P-labeled 0.8-kb *Eco*RI-*Bam*HI fragment that encodes approximately 84% of the extracellular domain of PRK1 (Mu et al. 1994). The membrane was washed twice for 10 min each time in ×0.1 SSC, 0.1% SDS at room temperature and then for 20 min at 60°C. The membrane was exposed on X-ray film at –70°C for 2 days with an intensifying screen.

Results

Microscopic examination of mature ovules and developing seeds of ASRK-13 and wild-type plants

We first examined the ovules isolated from open flowers of transgenic plant ASRK-13 and a wild-type plant by SEM. No obvious morphological differences could be detected at this level of resolution between the ovules of the wild-type plant (Fig. 1A) and ASRK-13 (Fig. 1D). We next pollinated ASRK-13 and the wild-type plant with wild-type pollen and examined by SEM the developing seeds at 3 DAP and 6 DAP. At 3 DAP, the ovary of ASRK-13 contained approximately equal numbers of large- and small-sized seeds (Fig. 1E), whereas almost all seeds of the wild-type plant were approximately the same size as the large-sized seeds of ASRK-13 (Fig. 1B). At 6 DAP, the size difference between the two size groups of the developing seeds of ASRK-13 became even more prominent (Fig. 1F). The small-sized seeds appeared flattened and to have "shrunk" in size relative to the small-sized seeds observed at 3 DAP, whereas the large-sized seeds had increased in size relative to the large-sized

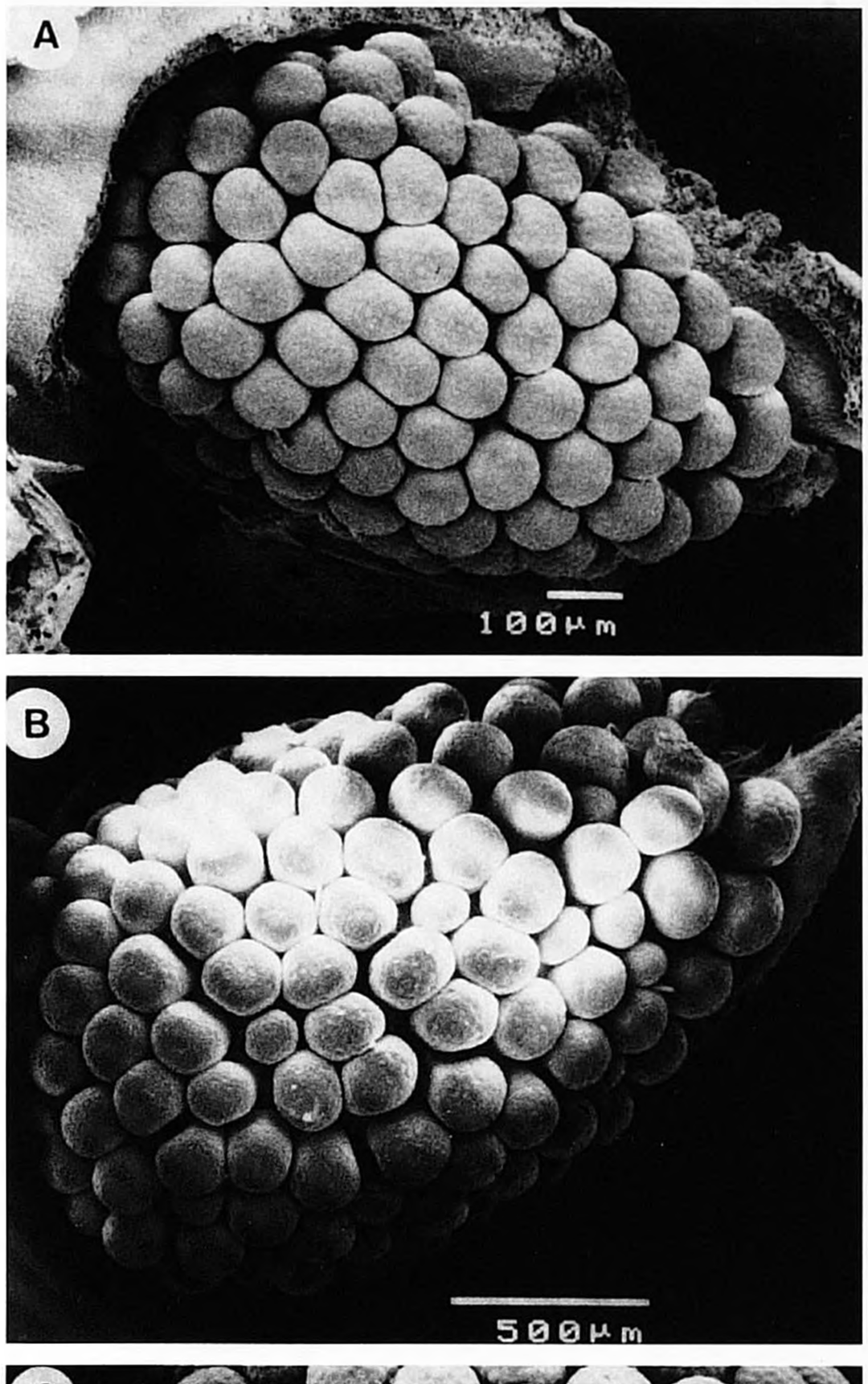


Fig. 1A–F Scanning electron micrographs of mature ovules and developing seeds of ASRK-13 and a wild-type plant. **A, D** Mature ovules from the wild-type plant (**A**) and ASRK-13 (**D**). **B, E** Developing seeds from the wild-type plant (**B**) and ASRK-13 (**E**) at 3 DAP. **C, F** Developing seeds from the wild-type plant (**C**) and ASRK-13 (**F**) at 6 DAP. In (**E**), a normal developing seed is marked *n* and an abnormal developing seed is marked *ab*; in (**F**), two of the aborted seeds are marked *ab*. For both ASRK-13 and the wild-type plant, ovules from 10–15 ovaries were examined for each developmental stage, and the numbers of normal and abnormal ovules (seeds) were counted under a light microscope after the ovules had been dissected from the ovary

seeds observed at 3 DAP. In the wild-type plant, more than 90% of the seeds were approximately the same size as the large-sized seeds of ASRK-13 (Fig. 1C).

To more closely study the developing seeds of ASRK-13, we examined longitudinal sections of the ovary of ASRK-13 at 6 DAP under a light microscope. Both small- and large-sized seeds were observed; the development of the former appeared to be aborted (Fig. 2A). Higher magnification of ovary sections indeed revealed

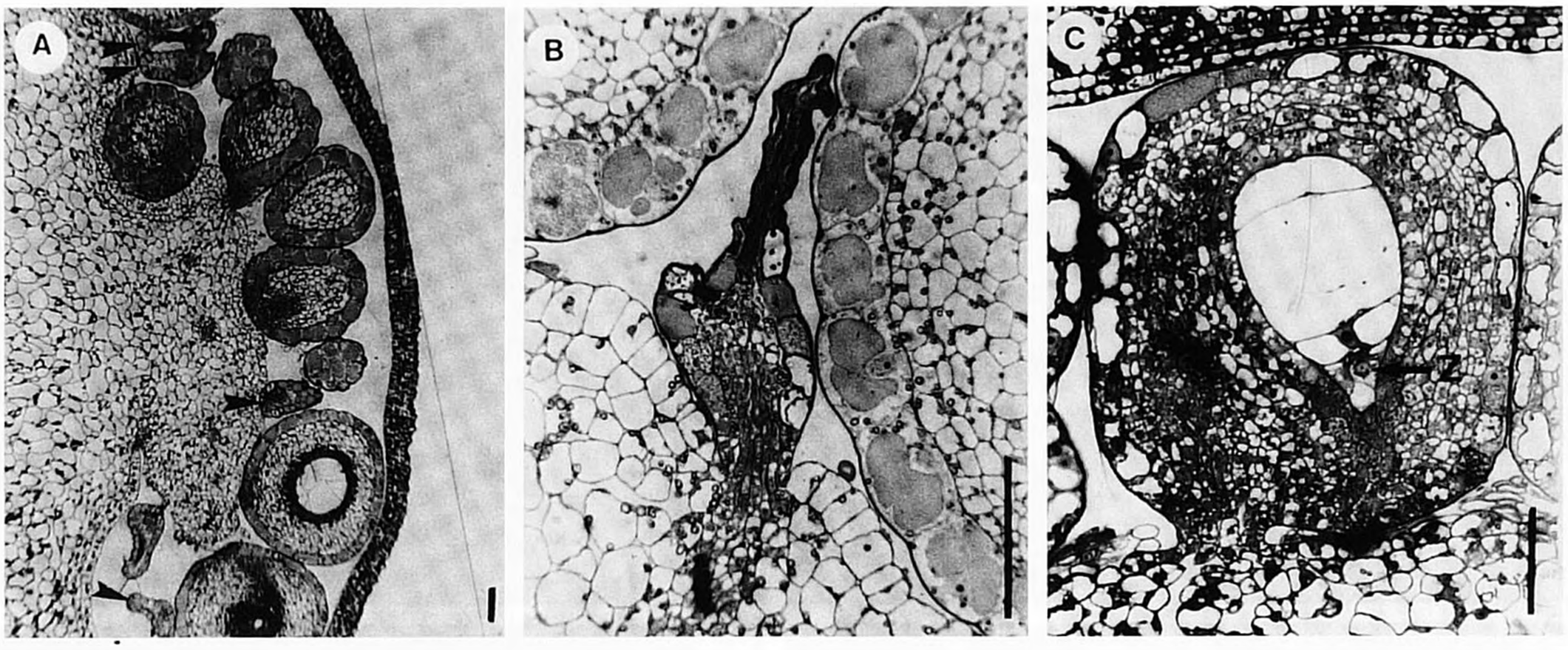


Fig. 2A–C Light micrographs of sections of ovaries and developing seeds of ASRK-13 and a wild-type plant. **A** Bright field micrograph of a cross section of an ovary of ASRK-13 at 6 DAP. **B** Bright field micrograph of a longitudinal section of an ovary from an aborted seed of ASRK-13 at 6 DAP. **C** Bright field micrograph of a cross section of a normally developed seed of a wild-type plant at 3 DAP. In (**A**), aborted seeds are marked with arrowheads; in (**C**), the zygotic embryo is marked *Z* and the endosperm has divided with one of the endosperm nuclei clearly visible. *Bars* = 50 μm

that the embryo sac as well as the sporophytic tissues of the small-sized seeds had degenerated (Fig. 2B).

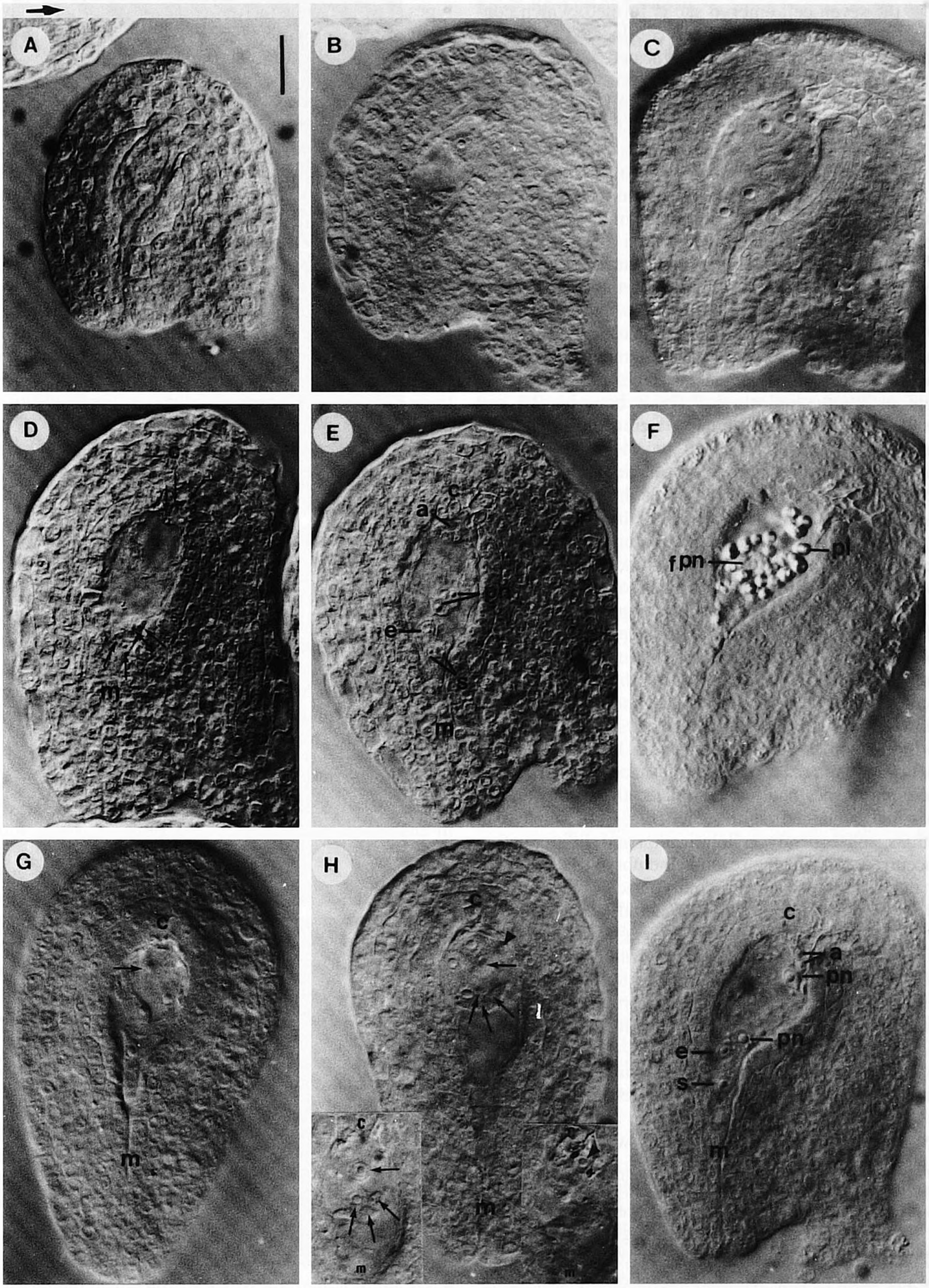
Because the size difference between the abnormal and normal developing seeds was apparent at 3 DAP,we also sectioned the ovary of the wild-type plant at 3 DAP to examine the stage of embryo development. As shown in Fig. 2C, the embryo was still at the zygote stage prior to the first mitosis, whereas the primary endosperm had already divided. These results suggest that the seed development of ASRK-13 was affected at a very early stage, raising the possibility that the failure of seed development might be caused by an abnormality in ovule development and/or fertilization.

Embryo sac development of ASRK-13 and wild-type plant

We previously divided the flower development of *P. inflata* into six stages; stages 1–5 (based on increasing size of flower buds) and the open flower stage (Mu et al. 1994). To examine the structure of the ovules of ASRK-13 and the wild-type plant at these flower developmental stages, we first dissected and cleared the ovules from the fixed ovaries in each developmental stage. We then used differential interference contrast microscopy to examine the size, location, and morphology of the embryo sac and the surrounding sporophytic tissues, as well as plastid formation in the embryo sac. A total of approximately 2000 ovules from ten ovaries of each plant were examined for each developmental stage. Because we did not detect any apparent abnormality in the development of the sporophytic tissues of the ovule from ASRK-13 (results not shown), only the development of the embryo sac is discussed below.

In *P. inflata*, embryo sac development follows the pattern of polygonum type (Willemse and van Went 1984; Reiser and Fischer 1993) and is divided into two stages: megasporogenesis and megagametogenesis. During megasporogenesis, a megasporocyte undergoes meiosis to produce four megaspores. The megaspore closest to the chalazal end undergoes three successive mitotic divisions, whereas the other three megaspores degenerate. After the first mitosis of the functional megaspore, the two nuclei migrate to opposite poles of the embryo sac. Each of the two nuclei undergoes two more rounds of mitosis, resulting in an eight-nucleate megagametophyte. After cellular-

Fig. 3A–I Embryo sac development of ASRK-13 and a wild-type plant. Ovules at different developmental stages were cleared with Herr's clearing fluid (Herr 1971) and observed under a differential interference contrast microscope. **A–F** Micrographs of ovules from the wild-type plant which contain uninuclear embryo sacs (**A**), a bi-nucleate embryo sac (**B**), a four-nucleate embryo sac (**C**), an eight-nucleate embryo sac (**D, E**), and a mature embryo sac (**F**). **G–I** Micrographs showing the three representative phenotypes of abnormal ovules from ASRK-13 at the open flower stage. The embryo sac shown in (**A**) contains a single functional nucleus at the chalazal end. In the embryo sac shown in (**D**), four of the nuclei are located at the micropylar end of the embryo sac, whereas the other four are located at the chalazal end. The *arrows* indicate the eight nuclei. In (**G**), embryo sac development was arrested at the four-nucleate stage. The *arrow* indicates a nucleus that is located at a different optical plane. In (**H**), embryo sac development was arrested at the eight-nucleate stage, and all of the nuclei were located at the chalazal end of the embryo sac. The two *inserts* show the same embryo sac pictured at two different optical planes. *Arrows* and the *triangle* indicate the nuclei located at different optical planes, which are visualized in the small photos. In (**I**) two polar nuclei were still located at opposite poles of the central cell. At this focal plane, one of the antipodal cells and one of the synergids are not visible. Abbreviations: *a* antipodal cells; *c* chalaza; *e* egg cell nucleus; *f* fused polar nuclei; *pl* plastids; *pn* polar nucleus or partially fused polar nuclei; *m* micropyle; *s* synergid nucleus. The *bar* for the photograph shown in (**A**) is 20 μm and all the other photographs were taken at the same magnification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ization, the seven-cell embryo sac is formed, which consists of two synergids and one egg cell located at the micropylar end, three antipodal cells located at the chalazal end, and a central cell (containing two polar nuclei) occupying the center of the embryo sac. Initially, the polar nuclei are located at the opposite poles of the large vacuole that is in the center of the central cell, but they subsequently migrate to the micropylar end of the central cell near the base of the egg apparatus to fuse with each other. Throughout the embryo sac, especially in the central cell, numerous plastids which contain starch are formed, and they become very large toward the mature stage of the embryo sac. Further differentiation of the cells follows and the embryo sac matures.

When embryo sac development of the wild-type plant was examined at the six flower developmental stages, the following results were obtained. At stage 1 (buds less than 0.5 cm in length) and stage 2 (buds between 0.5 and 1.0 cm in length), the integuments were initiated and the megaspore mother cell completed meiosis to produce four megaspores, with the three non-functional megaspores subsequently degenerating (results not shown). At stage 3 (buds between 1.0 and 1.5 cm in length), approximately 85% of the embryo sacs contained a uninucleate megaspore (Fig. 3A), but in some embryo sacs the megaspore had already completed the first mitosis to yield two nuclei (Fig. 3B). The embryo sacs at this stage were generally small and contained few plastids. At stage 4 (buds between 1.5 and 2.0 cm in length), most of the embryo sacs contained four nuclei (Fig. 3C), indicating that the megaspore had completed the second mitosis. Development of the plastids was variable; some embryo sacs contained more plastids than others. At stage 5 (buds between 2.0 and 2.5 cm in length, anthers containing mature pollen), some of the embryo sacs had just completed the final round of mitosis and contained eight nuclei (Fig. 3D), whereas in the others, subsequently, cellularization and differentiation had occurred, forming the seven-cell embryo sac (Fig. 3E). Polar nuclei in the seven-cell embryo sacs were either located at the opposite poles of the central cell (results not shown), or had migrated to near the egg apparatus at the micropylar end (Fig. 3E). In embryo sacs in which the polar nuclei had migrated, the polar nuclei were either located very close to each other, or were partially fused as in the embryo sac shown in Fig. 3E. The embryo sacs at this stage were larger than at earlier stages; the egg cell and synergids, in particular, had expanded and elongated. The formation of plastids was more prominent than at earlier stages. At the open flower stage, more than 90% of the ovules contained mature embryo sacs which were filled with large numbers of large plastids (Fig. 3F). The remaining ovules contained aborted embryo sacs. In many of the mature embryo sacs, the polar nuclei were fused and the three antipodal cells had degenerated (Fig. 3F).

Approximately half of the ovules of ASRK-13 followed the same pattern of embryo sac development as the ovules of the wild-type plant to produce normal embryo sacs, whereas the other half contained abnormal embryo sacs at the open flower stage with three different phenotypes. Two rare phenotypes showed embryo sac development arrested at either the four-nucleate stage (Fig. 3G) or the eight-nucleate stage (Fig. 3H), where somewhat irregular positioning of the nuclei was observed. The most frequently observed phenotype (approximately 80% of the abnormal ovules examined) is shown in Fig. 3I. The megaspore completed three rounds of mitosis and part of the maturation process to produce a seven-cell embryo sac, but subsequent developmental events appeared to have failed. The abnormal embryo sacs were typically narrower and smaller than the normal ones, and their synergids and egg cells failed to fully expand. Their polar nuclei were still located at the opposite poles in the central cell, indicating that nuclear migration had been hampered. Plastid formation in these abnormal embryo sacs was invariably poor. These results suggest that most of the mutant ovules of ASRK-13 were able to progress through most of megagametogenesis, but failed to complete the maturation stages of embryo sac development when cell expansion, nuclear migration, and differentiation take place.

Staining of pollen tubes inside ovules

To determine whether the abnormal ovules of ASRK-13 were fertilized, we used toluidine blue (at low concentration to block autofluorescence from ovules) and aniline blue to stain pollen tubes in the ovaries of ASRK-13 and the wild-type plant at 2 DAP, following the protocol of Smith and McCully (1978), and examined the stained pollen tubes under a fluorescence microscope. Twenty ovaries each from ASRK-13 and the wild-type plant were stained and 50 to 70 ovules from each ovary were examined to determine the percentage of unfertilized ovules. For ASRK-13, approximately half of the ovules in each ovary had distinctive staining of pollen tubes at their micropylar ends, indicating penetration of pollen tubes, whereas the other half lacked the staining (Fig. 4A). Figs. 4B and 4C show a higher magnification of an ovule penetrated by a pollen tube, and an ovule without pollen tube, respectively, at 2 DAP. Approximately 80% of the wild-type plant ovules had staining of pollen tubes at the micropylar end (results not shown). Using a differential interference contrast microscope, we also found that significantly more ovules of ASRK-13 than ovules of the wild-type plant lacked enlarged synergids (results not shown). Because synergids normally become enlarged after fusion with an entering pollen tube in the embryo sac, this observation is consistent with the pollen tube staining results. All the results taken together suggest that half of the ovules produced by ASRK-13, most likely the half that developed abnormally, could not be fertilized.

RNA gel blot analyses of *PRK1* mRNA in the ovary

We previously detected the *PRK1* message by RNA gel blot analysis in stage 3 through stage 5 anthers and in

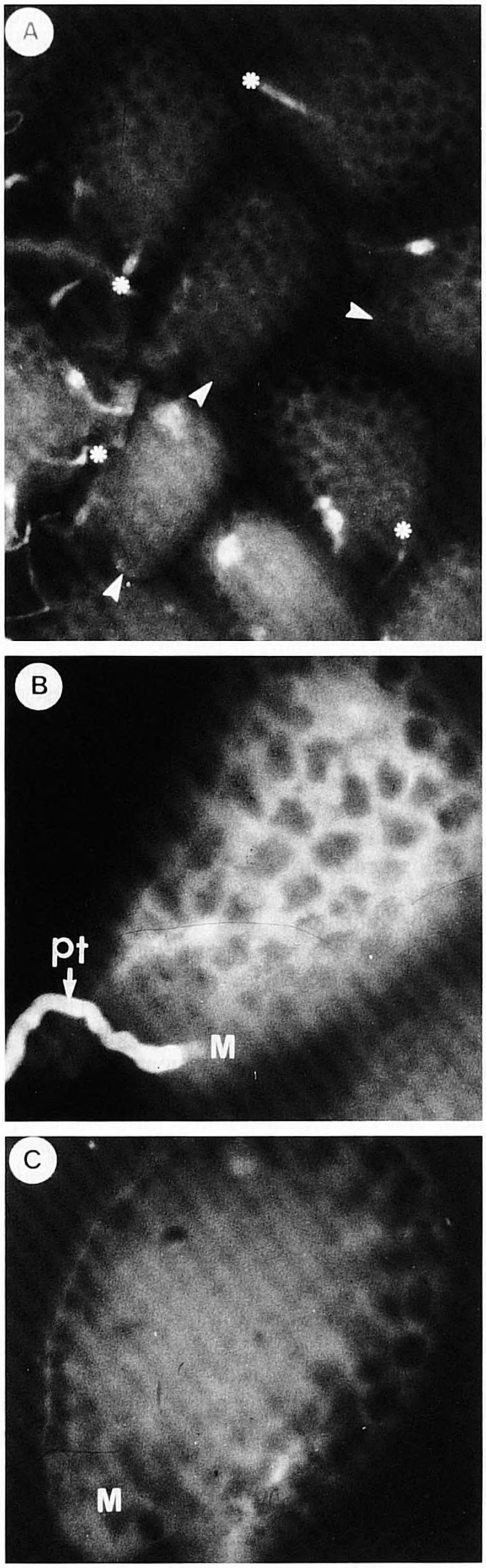

Fig. 4A–C Staining of pollen tubes in ovules. At 2 DAP, the ovaries from ASRK-13 were collected and stained with toluidine blue and aniline blue. A pollen tube fluoresces distinctively at the micropyle of the ovule after it enters the ovule. **A** Fluorescence micrograph of pollen tubes inside some of the ovules. **B** Fluorescence micrograph of a pollen tube (*pt*) entering the micropyle (*m*) of an ovule. **C** Fluorescence micrograph of an ovule with no pollen tube entry. *Asteriks* in (**A**) indicate micropyles where a pollen tube has entered and *arrowheads* indicate micropyles with no pollen tube entry

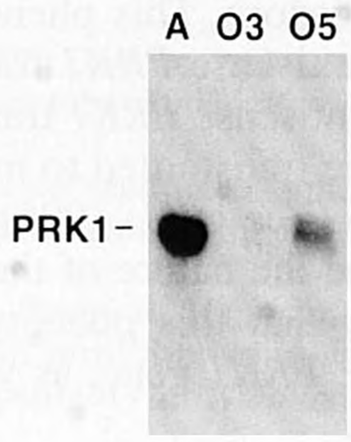

Fig. 5 RNA gel blot analysis of *PRK1* mRNA in ovary and anther tissues of ASRK-13. *Lane A* contains 30 µg of total RNA isolated from anthers of stage 5 buds; *lanes 03 and 05* contain 2 µg each of poly $(A)^+$ RNA isolated from ovaries of stage 3 and stage 5 buds, respectively

mature pollen, but failed to detect the message in all other tissues examined, including the pistil tissue. However, the pistil tissue used did not include the ovary. To investigate whether the *PRK1* gene is expressed in the ovary, we first carried out RNA gel blot analysis on equal amounts (100 µg) of total RNA isolated from the stage 3 and stage 5 ovaries and from the stage 5 anther. The expected RNA band was detected in the stage 5 anther, but no signal was detected in the stage 3 ovary and a faint band, barely detectable, was observed in the stage 5 ovary (results not shown). We then repeated the RNA gel blot analysis using poly $(A)^+$ RNA purified from the total RNA of the stage 3 and stage 5 ovaries, and the total RNA of the stage 5 anther. As shown in Fig. 5, no RNA band was detected in the stage 3 ovary, but an RNA band of approximately the same size as the *PRK1* message detected in the stage 5 anther was detected in the stage 5 ovary. The intensity of the *PRK1* mRNA in the stage 5 ovary was much lower than that of the *PRK1* mRNA detected in the stage 5 anther, despite the fact that poly $(A)^+$ RNA from the ovaries and total RNA from the anthers were used. These results suggest that the *PRK1* gene is expressed in the ovary, albeit at a much lower level than in the anther.

Discussion

The antisense transgenic plant, ASRK-13, of *P. inflata* studied here was previously generated for the purpose of studying the function of PRK1 in pollen development (Lee et al. 1996), because PRK1 was initially thought to be expressed exclusively in pollen (Mu et al. 1994). ASRK-13 and other transgenic plants were found to exhibit a pollen abortion phenotype, with microspore development initially arrested at the uninucleate stage followed by degeneration of the cytosolic contents of the

microspore. This phenotype was shown to be caused by the antisense *PRK1* transgene. During progeny analysis, the antisense *PRK1* transgene was unexpectedly found to not be transmitted to most of the progeny through the female (Lee et al. 1996). This finding prompted us to examine the nature of the female phenotype and the possibility that this phenotype was also caused by the antisense *PRK1* gene, as was the case for the pollen phenotype.

In this report, we show that the embryo sac development of one-half of the ovules produced by ASRK-13 is abnormal, with development arrested at the four- or eight-nucleate stage in some affected ovules, and in the seven-cell stage in most of the affected ovules. In the latter case, the developmental abnormalities include: (1) the embryo sacs are smaller and narrower than normal sacs; (2) the two polar nuclei in the central cell fail to migrate toward the egg apparatus and remain located at opposite poles of the cell; (3) the egg cells and synergids are not fully differentiated; and (4) the plastids are not fully developed. All of these abnormal features taken together suggest that the developmentally arrested embryo sacs of ASRK-13 either fail to complete mitosis or fail to complete the maturation stages of development. The ovules containing these mutant embryo sacs could not achieve fertilization and they subsequently degenerated during seed growth.

The most direct means to demonstrate the cause-and-effect relationship between the antisense *PRK1* transgene and the several phenotypes is to show that the level of *PRK1* mRNA in the ovary of ASRK-13 is reduced by 50% relative to the level of *PRK1* mRNA in the ovary of wild-type plants. (Since one-half of the ovules produced by ASRK-13 are normal, the maximum level of reduction is 50%.) However, our attempts to use RNA gel blot analysis to measure the degree of reduction of *PRK1* mRNA in the ovary did not yield conclusive results (not shown). This is largely because the level of *PRK1* mRNA in the ovary is very low even in wild-type plants and, thus, it was very difficult, if not impossible, to accurately quantify the level of *PRK1* mRNA in wild-type plants and ASRK-13.

Nonetheless, several lines of indirect evidence lend support to the notion that the arrest in embryo sac development likely results from the antisense *PRK1* transgene. First, we previously showed that ASRK-13 contained one active copy of the antisense *PRK1* transgene (Lee et al. 1996). Thus, only half of the megaspores produced by ASRK-13 are expected to inherit the active transgene. This is consistent with the observation that approximately half of the ovules had abnormal embryo sac development. Second, transgenic plants that did not exhibit the pollen abortion phenotype did not exhibit the female phenotype either. That is, those transgenic plants that carried inactive copies of the antisense *PRK1* transgene, as judged by their normal pollen development, had normal embryo sac development. Third, RNA gel blot analysis revealed that the *PRK1* gene is expressed in the ovaries of stage 5 flower buds of wild-type plants. Fourth, the *LAT52* promoter used to express the antisense *PRK1* gene has been shown to be active in mature and immature seeds (Twell et al. 1991), and transgenic tobacco plants carrying the *LAT52* promoter-diphtheria toxin fusion gene have been found to show greatly reduced transmission of the transgene through the female (Twell 1995).

The study of ovule development has been hampered by the inaccessibility of the tissue within the ovary. Recently, genetic and molecular approaches have been used to investigate the developmental processes, particularly in *Arabidopsis*, and various mutations that affect ovule development have been identified (Robinson-Beers et al. 1992; Pruitt et al. 1994; Ray et al. 1994; Hülskamp et al. 1995). However, most of the mutations identified are sporophytic in nature, due to the relative ease of screening. So far, only a few gametophytic mutations affecting embryo sac development have been described (Rédei 1964; Lin 1981; Springer et al. 1995). For example, the *indeterminate gametophyte* (*ig*) mutation of maize shows a pleiotrophic phenotype: multiple egg cells and embryos accumulated in the embryo sac, and defective kernels were formed (Kermicle 1971; Lin 1981). *Gf* mutations of *Arabidopsis* show reduced transmission of the genes linked to *Gf* through the female (Rédei 1964). In the *PROLIFERA* mutation generated by gene trap tagging, embryo sac development is arrested at various stages, resulting in abortion of gametophytes (Springer et al. 1995). However, to date, there is very little information on the molecular mechanisms that regulate the coordinated developmental processes involved in the formation of embryo sacs.

Unlike the mutations described above, the block in embryo sac development exhibited by ASRK-13 occurs, in most cases, at late stages of development, after the formation of the seven-cell embryo sac. The abnormal embryo sacs are able to progress through most of megagametogenesis, but their development stops short of the final maturation stages when cell wall formation, nuclear migration, and cell expansion and differentiation occur. If PRK1 is indeed involved in these late stages of embryo sac development, it must act gametophytically, as it does in pollen development, because development of only approximately one-half of the embryo sacs was affected by the antisense *PRK1* gene. Thus, PRK1 would add to the so far very small number of gametophytically acting genes that are known to be involved in ovule and/or female gametophyte development. In that capacity, PRK1 may serve as a transducer of an external signal, perhaps from sporophytic tissues of the ovule, to elicit a cascade of signal transduction events. These events would result in coordinated regulation of various cellular processes required for the completion of the embryo sac development.

To ultimately determine if and how PRK1 regulates the developmental processes, we have begun to identify the ligand(s) and substrate(s) of PRK1 using a yeast two-hybrid protein-protein interaction assay (Bartel et al. 1993). Preliminary results show that the kinase domain of

PRK1 interacts strongly with several pollen proteins, one of which appears to be a cytoskeletal protein (A.L. Skirpan and T.-h. Kao, unpublished results). It has been reported that the cytoskeleton of plant cells plays important roles in establishing and maintaining specific polarities and spindle formations (Baskin and Cande 1990), and in nuclear positioning, nuclear division, cytoplasm polarity, and cellularization during male and/or female gametophyte development (Gunning and Hardham 1982; Bednara et al. 1988; Willemse and van Lammeren 1988; Staiger and Cande 1990; Webb and Gunning 1990; Huang and Sheridan 1994). Thus, the possibility that PRK1 may interact directly with some cytoskeletal protein to regulate a broad spectrum of cellular activities required for microspore mitosis and embryo sac development is intriguing. Further study of the role of PRK1 in microspore mitosis and embryo sac development may provide a new insight into the molecular and biochemical mechanisms underlying these two developmental processes.

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